

**PHARMACOLOGICAL OPTIMIZATION OF THE
ANTIHYPERTENSIVE AND VASORELAXANT
EFFECTS OF A POLYHERBAL TRADITIONAL
CHINESE MEDICINE EXTRACT BASED ON
ORTHOGONAL STIMULUS-RESPONSE
COMPATIBILITY APPROACH.**

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UNIVERSITI SAINS MALAYSIA

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by

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LIST OF ABBREVIATIONS

1D-IR	1D-Infrared
2-APB	2-Aminoethoxydiphenyl Borate
2D-IR	2D-Infrared
4-AP	4-Aminopyridine
5-HT_{1D}	5-Hydroxytryptamine 1D Receptor
A/G	Albumin/Globulin Ratio
AA	Arachidonic Acid
AC	Adenylyl Cyclase
Ach	Acetylcholine
ADC 2	Automatic Developing Chamber 2
AHA	American Heart Association
Akt	Protein Kinase B
AlbP	Albumin Protein
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AMP	Adenosine Monophosphate
AO50	50% Ethanolic Extract Of <i>Alisma Orientale</i>
AO95	95% Ethanolic Extract Of <i>Alisma Orientale</i>
AOW	Distilled Water Extract Of <i>Alisma Orientale</i>
AR	Actual Relaxation
ARASC	Animal Research And Service Centre

AST	Aspartate Aminotransferase
AT₂	Angiotensin II Receptor Type 2
ATP	Adenosine Triphosphate
ATS 4	Automatic TLC Sampler 4
AU	Absorbance Units
B₂	Bradykinin Receptor 2
BaCl₂	Barium Chloride
BiID	Direct Bilirubin
BiliT	Total Bilirubin
BK_{ca}	Big-Conductance Calcium-Activated Potassium Channels
BP	Blood Pressure
BUSE	Blood Urea Serum Electrolyte
Ca²⁺	Calcium Ions
CALCRL	Calcitonin Receptor-Like Receptor
cAMP	Cyclic Adenosine Monophosphate
CBS	Cystathionine Beta Synthase
cGMP	Cyclic Guanosine Monophosphate
CICR	Calcium-Induced Calcium Release
Cl⁻	Chloride Ions
C_{max}	Maximum Contraction
CMC	Carboxymethyl Cellulose
COX	Cyclooxygenases
CSE	Cystathionine Gamma-Lyase

CYP	Cytochrome P450
DAG	Diacylglycerol
DMSO	Dimethyl Sulfoxide
EC₅₀	Half Of Maximum Effective Concentration
EDRFs	Endothelium-Derived Relaxing Factors
EETs	Epoxyeicosatrienoic Acid
EGTA	Ethylene Glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic Acid
eNOS	Endothelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
ET_BR	Endothelin Receptor Type B
FTIR	Fourier Transform Infrared
G protein	Guanine Nucleotide-Binding Proteins
GDP	Guanosine Diphosphate
GE50	50% Ethanolic Extract of <i>Gastrodia Elata</i>
GE95	95% Ethanolic Extract of <i>Gastrodia Elata</i>
GEF	Guanine Nucleotide Exchange Factor
GEW	Distilled Water Extract of <i>Gastrodia Elata</i>
GGT	Gamma-Glutamyl Transferase
GMP	Guanosine Monophosphate
GPCRs	G Protein-Coupled Receptors
GTP	Guanosine Triphosphate
H₂S	Hydrogen Sulfide
HCT	Hematocrit

HGB	Hemoglobin
HPTLC	High Performance Thin Layer Chromatography
IK_{ca}	Intermediate-Conductance Calcium-Activated Potassium Channel
iNOS	Inducible Nitric Oxide Synthase
IP	Prostacyclin Receptor
IP₃	Inositol Triphosphate
IP₃R	Inositol Triphosphate Receptor
JNC 7	Seventh Report Of The Joint National Committee
K⁺	Potassium Ions
K_{ATP}	ATP-Sensitive Potassium Channels
KBr	Potassium Bromide
K_{ca}	Calcium-Activated Potassium Channels
K_{ir}	Inwardly-Rectifying Potassium Channels
Krebs'	Krebs-Henseleit Solution
K_v	Voltage-Operated Potassium Channels
LFT	Liver Functional Test
L-NAME	L-N ^G -Nitro Arginine Methyl Ester
M₃	Muscarinic Acetylcholine Receptor 3
MAP	Mean Arterial Pressure
MB	Methylene Blue
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Cell Volume
MLCK	Myosin-Light-Chain Kinase

MLCP	Myosin-Light-Chain Phosphatase
Na⁺	Sodium
nNOS	Neuronal Nitric Oxide Synthase
NSAID	Non-Steroidal Anti-Inflammation Drugs
ODQ	1H-[1,2,4] Oxadiazolo [4,3-a]Quinoxalin-1-One
pD₂	Negative Logarithm To Base 10 Of The EC50
PDE	Phosphodiesterase
PE	Phenylephrine
PGH₂	Prostaglandin H ₂
PIP₂	Phosphatidylinositol 4,5-Bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PLA₂	Phospholipase A ₂
PLC	Phospholipase C
PLT	Platelet
PN50	50% Ethanolic Extract Of <i>Panax Notoginseng</i>
PN95	95% Ethanolic Extract Of <i>Panax Notoginseng</i>
PNW	Distilled Water Extract Of <i>Panax Notoginseng</i>
PT50	50% Ethanolic Extract Of <i>Pueraria Thomsonii</i>
PT95	95% Ethanolic Extract Of <i>Pueraria Thomsonii</i>
PTW	Distilled Water Extract Of <i>Pueraria Thomsonii</i>
RBC	Red Blood Cell

R_f	Retention Factor
RFT	Renal Functional Test
R_{max}	Maximum relaxation
ROCC	Receptor-Operated Calcium Channel
RyRs	Ryanodine Receptor
SD	Standard Deviation
SD-IR	Second-Derivative Infrared
SERCA	Sacro/Endoplasmic Reticulum Ca ²⁺ -ATPase
sGC	Soluble Guanylyl Cyclase
SHRs	Spontaneous Hypertensive Rats
SK_{ca}	Small-Conductance Calcium-Activated Potassium Channels
SR	Sacroplasmic Reticulum
TCM	Traditional Chinese Medicine
TP	Total Protein
TRPV₄	Transient Receptor Potential Cation Channel Subfamily V
TXA₂	Thromboxane A ₂
UR50	50% Ethanolic Extract Of <i>Uncaria Rhynchophylla</i>
UR95	95% Ethanolic Extract Of <i>Uncaria Rhynchophylla</i>
URW	Distilled Water Extract Of <i>Uncaria Rhynchophylla</i>
VOCC	Voltage-Operated Calcium Channels
VSMCs	Vascular Smooth Muscle Cells
WBC	White Blood Cell
WHO	World Health Organization

LIST OF SYMBOLS

%	percentage
°C	degree Celsius
$10^9/l$	10^9 cells per liter
$10^{12}/l$	10^{12} cells per liter
$\mu g/ml$	microgram per liter
μl	microliter
μM	micromolar
$\mu mol/l$	micromole per liter
fl	femtoliter
g	gram
g/dl	gram per deciliter
g/l	gram per liter
mg	milligram
mg/ml	milligram per liter
mM	millimolar
mm	millimeter
mmHg	millimeter of mercury
mmol/l	millimole per liter
pg	picogram
rpm	rotation per minute
U/l	units per liter

**PENGOPTIMUMAN KESAN ANTIHIPERTENSIF DAN VASORELAKSAN
SECARA FARMAKOLOGI BAGI EKSTRAK UBATAN TRADISIONAL CINA
POLIHERBA BERDASARKAN PENDEKATAN KESERASIAN TINDAK
BALAS RANGSANGAN ORTOGONAL**

ABSTRAK

Hipertensi merupakan salah satu masalah kesihatan yang utama dan berkaitan dengan pelbagai penyakit seiring dan komplikasi, kebanyakan komplikasi berlaku pada buah pinggang serta sistem kardiovaskular dan hal ini telah mendapat perhatian saintis di seluruh dunia kebelakangan ini. Walaupun terdapat ubat-ubatan antihipertensi sintetik di pasaran, efikasi yang rendah dalam kaedah monoterapi, serta kesan sampingan kerap dilaporkan. Oleh itu, kajian ini bertujuan mewujudkan satu formula antihipertensi baru dengan menggunakan teori gabungan sindromik-penyakit yang baharu daripada kaedah perubatan tradisional Cina (TCM). Pemilihan herba TCM untuk kajian ini iaitu *Gastrodia elata*, *Uncaria rhynchophylla*, *Pueraria thomsonii*, *Panax notoginseng*, dan *Alisma orientale* adalah berdasarkan keupayaan herba-herba ini dalam merawat tiga sindrom utama tekanan darah tinggi seperti api, pengekalan cecair kahak, dan sindrom-sindrom difisiensi. Keutuhan kelima-lima herba mentah telah disahkan oleh kromatografi lapisan nipis berprestasi tinggi (HPTLC) dan kaedah spektroskopi inframerah dengan teknik pembetulan Fourier tiga fasa (FTIR) sebagai pengesahan cap jari sebelum eksperimen. Kesan vasodilatori kesemua ekstrak telah disiasat dengan menggunakan asai *in vitro* cincin aortic terpencil. Ekstrak etanolik 95% *U. rhynchophylla* (UR95), *P. thomsonii* (PT95), dan *P. notoginseng* (PN95), serta ekstrak etanolik 50% *G. elata* (GE50), dan *A. orientale* (AO50) didapati sebagai ekstrak yang

paling kuat dalam pendorongan kesan vasodilatori berbanding dengan ekstrak dalam pelarut lain. Oleh itu, ekstrak ini telah digunakan untuk kajian keserasian kumpulan ortogonal rangsangan-tindakan, dan nisbah yang terbaik (F1) didapati pada EC₀, EC₂₅, EC₂₀, EC₂₀ dan EC₁₀ masing-masing adalah GE50 UR95, PT95, PN95, dan AO50. Selain itu, ekstrak (F1-2) lima herba mentah yang telah disediakan dalam nisbah F1 dan diekstrak dalam etanol sebanyak 50% seterusnya disahkan sebagai ekstrak yang paling kuat dalam pameran kesan vasodilatori berbanding dengan ekstrak yang lain melalui nilai EC₅₀ dan R_{max} pada 0.028±0.005 mg/ml dan 101.71±3.64%. Kesan vasodilatori F1-2 adalah disebabkan tetapi bukan sahaja terhadap kepada isorinkofilin, rinkohfilin, puerarin, ginsenosida Rg1 dan Rb1, serta asitat alisol b melalui NO/sGC/cGMP, adrinerjik-β₂ dan reseptor-M₃, serta mekanisme rangkaian isyarat K_{ca}, K_v, K_{ir}, K_{ATP}, VOCC dan IP₃R. Kesan antihipertensi F1-2 telah ditentukan dengan lebih lanjut secara *in vivo* selama 28 hari dengan penyuapan sub-kronik dalam tikus hipertensi spontan (SHRs). Hasil kajian menunjukkan penurunan yang ketara dalam tekanan darah SHR berbanding dengan kumpulan kawalan bergantung kepada dos, manakala tidak menyebabkan apa-apa kesan sampingan di darah elektrolit urea serum, fungsi buah pinggang, fungsi hati, kiraan darah lengkap, dan kiraan pengkamiran tikus SHR walaupun sehingga dos yang tinggi (970.92 mg/kg) penyuapan F1-2 dalam model haiwan. Secara muktamadnya, F1-2 dijumpai sebagai berjaya dan berpotensi sebagai ubat anti-hipertensi dalam penggunaan ke atas manusia pada masa hadapan.

**PHARMACOLOGICAL OPTIMIZATION OF THE ANTIHYPERTENSIVE
AND VASORELAXANT EFFECTS OF A POLYHERBAL TRADITIONAL
CHINESE MEDICINE EXTRACT BASED ON ORTHOGONAL STIMULUS-
RESPONSE COMPATIBILITY APPROACH**

ABSTRACT

Hypertension is one of the major health concerns that are related to various concomitant diseases as well as complications that mainly occur in kidney and cardiovascular system and it has gained the attention of worldwide scientists in recent. Despite the availability of synthetic anti-hypertensive drugs in the market, the low efficacies in monotherapy, as well as adverse effects are frequently being reported. Hence, the current study decided to establish a new antihypertensive formula by applying a new syndromic-disease combination theory of traditional Chinese medicine (TCM). The selection of TCM herbs for current study which includes *Gastrodia elata*, *Uncaria rhynchophylla*, *Pueraria thomsonii*, *Panax notoginseng*, and *Alisma orientale* was based on their abilities to treat three main syndromes of hypertension such as fire, phlegm fluid retention, and deficiency syndromes. The authenticity of the five raw herbs was verified by high performance thin layer chromatography (HPTLC) and tri-step Fourier transform infrared (FTIR) fingerprints identification methods prior to the experiments. The vasodilatory effects of all extracts were investigated by using the *in vitro* isolated aortic rings assays. The 95% ethanolic extracts of *U. rhynchophylla* (UR95), *P. thomsonii* (PT95), and *P. notoginseng* (PN95), as well as 50% ethanolic extracts of *G. elata* (GE50), and *A. orientale* (AO50) were found as the most potent extracts for exerting vasodilatory effects compared to other solvent extracts. Hence,

these extracts were used for orthogonal stimulus-response compatibility group studies, and the best ratio (F1) was found at EC₀, EC₂₅, EC₂₀, EC₂₀, and EC₁₀ for GE50, UR95, PT95, PN95, and AO50, respectively. Moreover, the extract (F1-2) of five raw herbs which were prepared in F1 ratio and extracted in total by 50% ethanol was further confirmed as the most potent extract in exhibiting vasodilatory effects compared to any other extracts at EC₅₀ and R_{max} values of 0.028 ± 0.005 mg/ml and $101.71 \pm 3.64\%$, respectively. The vasodilatory effects of F1-2 were elicited by but not limited to isorhynchophylline, rhynchophylline, puerarin, ginsenosides Rg1 and Rb1, and alisol b acetate through NO/sGC/cGMP, β_2 -adrenergic and M₃-receptors, and also K_{ca}, K_v, K_{ir}, K_{ATP}, VOCC, and IP₃R signaling mechanism pathways. The antihypertensive effects of F1-2 were further determined *in vivo* by 28 days sub-chronic oral administration in spontaneous hypertensive rats (SHRs). Results showed significant decrease of SHRs blood pressure compared to negative control in dose-dependent manner, whilst without causing any adverse effects in the blood urea serum electrolyte, renal function, liver function, full blood count, and differential count of the SHRs even until the high dose (970.92 mg/kg) of F1-2 administration. Conclusively, F1-2 was discovered to be a successful and could be a potentially useful antihypertensive drug for future use on humans.

CHAPTER 1

MICROENVIRONMENT OF THE BLOOD VESSELS IN VASCULAR TONE REGULATION

1.1 Introduction

Hypertension is a well-known “silent killer” and is one of the major risk factors of cardiovascular diseases. It is defined as persistently high blood pressure (BP) exerted against the wall of the arteries. There are approximately 13% of human populations in the earth suffering from hypertension and majority of them are from developing countries. In 2013, there was more than 32.7% of Malaysian aged ≥ 18 years old, and 43.5% of Malaysian aged ≥ 30 years old suffering from hypertension (MOH, 2013). The World Health Organization (WHO) has rated the hypertension as one of the most deadly causes of premature deaths worldwide. Hypertension is asymptomatic, it can live in anyone of us without causing any symptoms, and lead to concomitant diseases after many years, such as stroke.

Hypertension is categorized into several stages according to the American Heart Association (AHA) and Joint National Committee 7 (JNC 7). Normal BP stage has systolic pressure ≤ 120 mmHg and diastolic pressure ≤ 80 mmHg; pre-hypertension stage has systolic pressure ranging between 120-139 mmHg or diastolic pressure ranging between 80-89 mmHg; stage 1 hypertension has systolic pressure between 140-159 mmHg or diastolic pressure between 90-99 mmHg; and stage 2 hypertension has systolic pressure ≥ 160 mmHg or diastolic pressure ≥ 100 mmHg. In addition, a person with

systolic pressure of ≥ 180 mmHg or diastolic pressure ≥ 110 mmHg are considered as critical and should be treated immediately as medical emergency.

Generally, there are three types of blood vessels which includes the artery, vein, and capillary, they are responsible in transporting blood throughout the whole body. There are three layers present in artery and vein, where the outer most layer known as tunica adventitia, middle layer as tunica media, and innermost layer as tunica intima. Tunica media is rich of vascular smooth muscle cells (VSMCs), whereas tunica intima is composed of a thin layer of endothelial cells. Both the VSMCs and endothelium are the place where the vasomotors are located (Jakala et al., 2009, Yildiz et al., 2013). From the literatures, different parts of blood vessels were used by different researchers for vasculatures studies. However, based on the statistical analysis tabulated from 2010-2015, there were approximately 67% of the researches who recommend the use of aorta as the “golden tool” for pharmacological research in regards to vasculature, in order to minimize the orientation change of VSMCs and reduce the probability of causing damage to the endothelium (Loh et al., 2016). Generally, the vasomotors can cause the vascular tone to react in two ways such as vasodilation or vasoconstriction where both reactions are strictly dependent on the dominancy of the receptors present on the vascular endothelium and VSMCs as well as the interactions between their signals.

1.2 Endothelium-Derived Relaxing Factors (EDRFs)

There are two well-characterized EDRFs present in endothelium such as nitric oxide (NO) and prostacyclin (PGI₂). Additionally, the endothelium-derived hyperpolarizing factor (EDHF), and hydrogen sulfide (H₂S) have been claimed to be one of the EDRFs as well recently.

1.2.1 Nitric oxide (NO)

Generally, the NO can be produced by three isoform of NO synthase (NOS) such as neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). In endothelium, the increase in the concentration of the calcium in cytosol will enhance the formation of calcium-calmodulin complexes, which will activate the calmodulin-binding domain of the eNOS, and causes NO production. Other than that, the increase in hemodynamic shear stress, protein kinase A (PKA), and protein kinase B (Akt) in the blood vessel will induce the phosphorylation of the eNOS at Ser1179 site (Quillon et al., 2015, Zhao et al., 2015). Once the eNOS is activated, it will catalyze the breakdown of L-arginine into NO. Subsequently, NO will diffuse into adjacent VSMCs to stimulate the activity of the components down its signaling cascade such as soluble guanylyl cyclase (sGC), cyclic guanosine monophosphate (cGMP), and protein kinase G (PKG), hence resulting in vasodilation (Jakala et al., 2009, Moncada, 2007). The production of the NO can further stimulate the K_{ca} channels (Bolotina et al., 1994, Li et al., 1997) and voltage-activated K^+ channels (K_v) through the sGC-independent pathway (Yuan et al., 1996). Regarding the mechanism study, the selective antagonist of eNOS, L- N^G -Nitro arginine methyl ester (L-NAME) is frequently used due to its low toxicity, and more solubility at neutral pH compared to other inhibitors (Balligand et al., 1993, Pfeiffer et al., 1996).

1.2.2 Prostacyclin (PGI_2)

It is also known as prostaglandin I₂, and is well-known as one of the important EDRFs which is capable of inhibiting platelet aggregation. In endothelium, the precursor of the PGI_2 is arachidonic acid (AA) which ordinarily exists in the phospholipid bilayer of membranes. The AA will be released into the cytosol either catalyzed by

phospholipase A₂ (PLA₂) or diacylglycerol (DAG) lipase by breaking down the phospholipid. The free mobile AA will be converted into prostaglandin H₂ (PGH₂) by cyclooxygenase (COX). Subsequently, the prostacyclin synthase will catalyze the breakdown of PGH₂ into PGI₂, whilst some of the PGH₂ is converted into thromboxane (TXA₂) by thromboxane synthase. Both the TXA₂ and PGI₂ functions are physiologically antagonize. The PGI₂ will bind to the prostacyclin receptor (IP) which is located on the membrane of the VSMCs. IP is a G_sα protein-coupled receptor, once the G_sα protein is bound to guanosine triphosphate (GTP), the membrane-bound adenylyl cyclase (AC) will be stimulated to convert the adenosine triphosphate (ATP) into 3',5'-cyclic adenosine monophosphate (cAMP), which will then activate the PKA, and causes vasodilatory effects (Berumen et al., 2012, Nichols and Nichols, 2008). In the mechanism studies, the non-selective COX inhibitor, indomethacin which is a non-steroidal anti-inflammatory drug (NSAID) is frequently used compared to others such as ibuprofen, meclofenamic acid, and diclofenac. Indomethacin is preferred because it can bind rapidly to the COX with high-affinity, is time-dependent and has slow reversibility (Dannhardt and Kiefer, 2001, Smith et al., 2000).

1.2.3 Endothelium-derived hyperpolarizing factors (EDHFs)

EDHFs were discovered when there were residual of endothelium-dependent relaxation observed even after the depletion of both NO and PGI₂ (Scotland et al., 2005, Wang, 2002). It is a kind of electrical signal that originated from the endothelium, which could subsequently induce the hyperpolarizing current in adjacent VSMC and cause vasodilatory effect. In vasculature, EDHF can be generated through various kinds of the reactions that happens between different biochemical components. For instance, part of the AA in the endothelium could be broken down into epoxyeicosatrienoic acids (EETs)

by cytochrome P450 (CYP) epoxygenase, at which the EETs could activate the small-conductance Ca^{2+} -activated K^+ channels (SK_{ca}) and intermediate-conductance Ca^{2+} -activated K^+ channels (IK_{ca}) which is located on the endothelium, hence causing the K^+ ions efflux, and induce the hyperpolarizing current that will be passed onto the adjacent VSMCs via the myoendothelial gap junction, which was electrically-coupled between both endothelial cells and VSMCs, and ultimately causing the VSMCs hyperpolarization and relaxation. Furthermore, the EETs can activate the transient receptor potential vanilloid 4 (TRPV4) that is located on the endothelium to allow Ca^{2+} influx into the cytosol, and subsequently causes an increase of intracellular calcium concentration (Ca^{2+} spark) due to the calcium-induced calcium release (CICR) reaction when the influx cytosolic Ca^{2+} stimulates the ryanodine receptors (RyRs) that are located on the sarcoplasmic reticulum (SR). However, in terms of action potential, the efflux of K^+ ions into the myoendothelial space leads to an increase in concentration of K^+ , causing the activation of the inwardly-rectifying K^+ channels (K_{ir}) on the VSMCs to allow the influx of K^+ ions back into the cytosol of VSMCs, and followed by efflux of K^+ ions through the big-conductance Ca^{2+} -activated K^+ channels (BK_{ca}) to extracellular and causes hyperpolarization, resulting in vasodilatory effect (Pires et al., 2013, Quyyumi and Ozkor, 2006).

1.2.4 Hydrogen sulfide (H_2S)

H_2S has a similar chemical profile as NO in vasculature. In the endothelium, the H_2S is produced from the L-cysteine which is catalyzed by cystathionine γ -lyase (CSE) and/or cystathionine β -synthase (CBS). The H_2S is capable of activating the IK_{ca} and SK_{ca} channels in endothelium, hence producing the hyperpolarizing current. Literatures stated that the production of H_2S could be increased at least two-fold in endothelium

when triggered by the VEGF and muscarinic receptors activation through the Ca^{2+} -calmodulin-dependent activation of the CSE (Coletta et al., 2012, Wang, 2009). Moreover, H_2S is diffusible into the adjacent VSMCs to inhibit the ATP from binding with ATP-sensitive K^+ channels (K_{ATP}), hence activating the channels and subsequently allowing the K^+ efflux which results in hyperpolarization. H_2S is also capable of inhibiting the phosphodiesterase 5 (PDE5) from breaking down the cGMP, hence enhances the vasodilatory effects.

1.3 Enzyme-Linked Receptors

These receptors can be called catalytic receptors which are located on the membrane. These receptors are functionally activated by catalytic enzymes and ligand-receptors. In vasculature, the guanylyl cyclase and serine-threonine protein kinases are the major enzyme-linked receptors that play the roles in vascular tone regulation.

1.3.1 Soluble guanylyl cyclase (sGC)

As aforementioned, the sGC is freely mobile in the cytosol of VSMCs which will be activated once the NO comes to bind with the heme group of sGC. Once the sGC is activated, it will cleave the GTP into cGMP, subsequently activates the PKG, and causes vasodilatory effect (Horowitz et al., 1996, Ko et al., 2008). The 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one (ODQ) is soluble in dimethyl sulfoxide (DMSO) and was reported to inhibit the sGC by oxidizing the heme group of the sGC (Moro et al., 1996, Olson et al., 1997, Schrammel et al., 1996) and it is more frequently being used compared to the other cGMP lowering agent, methylene blue (MB) (Kontos and Wei, 1993, Mayer et al., 1993).

1.3.2 Serine-threonine protein kinases

It is basically a group of kinase enzymes that is functionally activated when bound to their respective secondary messengers, and subsequently phosphorylates the hydroxyl (OH) group of serine or threonine side chain of proteins which results in physiological effects. In vascular tone regulation, the protein kinases involved are PKA, PKC, and PKG.

1.3.2(a) Protein kinase A (PKA)

PKA is also known as the cAMP-dependent protein kinase which is functionally activated once bound with cAMP. Once bound with cAMP, its detaching catalytic subunits will phosphorylates the serine or threonine sites of its substrate proteins, and causes vasodilatory effect, whilst the cAMP will be broken down into adenosine monophosphate (AMP) by phosphodiesterase 3 (PDE3) (Bowen and Haslam, 1991). Regarding this mechanism study on cAMP-dependent PKA pathway, the Rp-cAMPs is commonly used due to its cell-permeability, resistance to PDE degradation as well as its selectivity towards PKA (Bouschet et al., 2003). Additionally, the selective inhibitor for PDE 3 that is commonly used is milrinone.

1.3.2(b) Protein kinase C (PKC)

In both vascular endothelium and VSMCs, the PKC will be activated by DAG and binds with Ca^{2+} ions at C1 and C2 domain, respectively (Huang, 1989). Once the PKC is activated, it will phosphorylate the serine or threonine sites of its substrate proteins, hence causes vasoconstriction. The most commonly used blockers for PKC is BIM due to its high permeability and selectivity.

1.3.2(c) Protein kinase G (PKG)

As aforementioned, the PKG will be functionally activated once the cGMP binds to the regulatory units of the PKG without causing enzyme dissociation. After that, the activated catalytic units of the enzyme will phosphorylates the serine or threonine sites of its substrate proteins, and causes vasodilatory effect (Wall et al., 2003), whilst the cGMP will be broken down into guanosine monophosphate (GMP) which is catalyzed by PDE 5 (Paul and Snyder, 2012). The most commonly used cGMP-dependent PKG inhibitor is Rp-8-Br-PET cGMPs due to its high stability and the ability to block both PKG 1 and PKG 2 (Valtcheva et al., 2009). Additionally, the selective inhibitors for PDE 5 commonly used are sildenafil, dipyridamole, zaprinast and T-1032.

1.4 G Protein-Coupled Receptors (GPCRs)

GPCRs are also known as the seven-transmembrane domain receptors which are functionally activated when bound to its ligands to transmit the signals from the outside of the cell into its interior. Generally, there are three types of guanine nucleotide binding protein (G protein) subunits such as $G\alpha$, $G\beta$, and $G\gamma$ proteins, and the former is the one who plays the major role in vascular tone regulation which is further classified into at least three main sub-types such as $G_q\alpha$, $G_i\alpha$, and $G_s\alpha$. The activation of the G protein will be initiated once the GPCRs bind to its ligand which subsequently causes the G protein temporary acting as guanine nucleotide exchange factor (GEF) by changing its conformation, and exchange its guanosine diphosphate (GDP) into GTP. Once bound with GTP, the G protein trimer will be dissociated into $G\alpha$ -GTP monomer and $G\beta\gamma$ dimer. $G\alpha$ -GTP monomer will start to interact with their intracellular proteins for signal transduction and will be discussed in below session (Walaas et al., 1992), whereas $G\beta\gamma$ dimer will tend to activate certain types of signaling molecules including ion channels,

lipid kinases, phospholipases as well as its own signaling cascades (Dorsam and Gutkind, 2007, Yuen et al., 2010).

1.4.1 $G_q\alpha$ protein-coupled receptors

In vasculature, the activation of the $G_q\alpha$ protein will cleave the phosphatidylinositol 4,5-bisphosphate (PIP_2) into second messengers, inositol triphosphate (IP_3) and DAG by binding its $G_q\alpha$ -subunit to the phospholipase C (PLC). The IP_3 is soluble and diffusible in cell and binds to the intracellular receptor, IP_3 receptor (IP_3R) which is located inside the cell and on the sarcoplasmic reticulum (SR) to trigger the intracellular release of the Ca^{2+} ions from the SR into the cytosol. Whereas, the DAG will activate the PKC and result in the increase of Ca^{2+} concentration in cytosol. In vasculature, the $G_q\alpha$ protein-coupled receptors that are present in endothelium includes angiotensin-2 receptor (AT_2), serotonin receptor ($5-HT_{1D}$), bradykinin receptor (B_2), muscarinic-3 receptor (M_3), endothelin-B receptor ($ET_B R$), and calcitonin receptor-like receptor (CALCRL). Whereas, there are α_1 -adrenergic receptor (α_1), M_3 receptor, angiotension-1 receptor (AT_1), endothelin receptors ($ET_A R$ & $ET_B R$), serotonin receptor ($5-HT_2$), and thromboxane receptor (TXA_2) present in VSMCs (Bockaert et al., 2006, Chen et al., 2000, de Gasparo et al., 2000, Goodman et al., 2001, Ishii and Kurachi, 2006, Jakala et al., 2009, Klabunde, 2011, Yildiz et al., 2013).

1.4.2 $G_i\alpha$ protein-coupled receptors

In vasculature, there is a $G_i\alpha$ protein-coupled receptor present in the VSMCs such as α_2 -adrenergic receptor (α_2). Once this receptor is activated by its agonist, it will inhibit the activity of the cAMP-dependent AC to convert ATP into cAMP, hence causes vasoconstriction (Klabunde, 2011, Qin et al., 2008).

1.4.3 G_sα protein-coupled receptors

Typically, the G_sα protein-coupled receptor is functionally opposed to G_iα protein-coupled receptor, where it will activate AC to produce cAMP from ATP, the increase in the production of cAMP will subsequently enhance the activation of PKA and results in vasodilatory effect. There are at least two major G_sα protein-coupled receptors present in VSMCs such as β₂-adrenergic receptor (β₂) and PGI₂ receptor (IP) (Jakala et al., 2009, Klabunde, 2011). In addition, the commonly used AC inhibitor for mechanism study is SQ22536 because it is more selective towards AC, higher cell-permeability and better solubility.

1.5 Channel-Linked Receptors

These channels are also known as ion channel-linked receptors and/or ligand-gated receptors and/or ionotropic receptors. These receptors will be functionally activated when bound to its ligand and allowing ions such as sodium (Na⁺), potassium (K⁺), chloride (Cl⁻), and calcium (Ca²⁺) move through the membrane. In vasculature, the action potential which occurs in VSMCs will be regulated by these receptors through hyperpolarization or depolarization. There are two important channel-linked receptors playing major roles in vascular tone regulation which are K⁺ channels and Ca²⁺ channels.

1.5.1 Potassium channels (K⁺)

K⁺ channel is the most widely distributed types of ion channel in living organisms (Littleton and Ganetzky, 2000). Typically, there are four types of K⁺ channels frequently discussed and investigated in vascular tone regulation which includes calcium-activated K⁺ channel (K_{ca}), voltage-gated K⁺ channel (K_v), ATP-sensitive K⁺ channel (K_{ATP}), and inwardly-rectifying K⁺ channel (K_{ir}).

1.5.1(a) Calcium-activated K^+ channel (K_{ca})

In human vasculature, K_{ca} channel can be further divided into three subtypes including big-conductance K_{ca} channel (BK_{ca}), intermediate-conductance K_{ca} channel (IK_{ca}), and small-conductance K_{ca} channel (SK_{ca}). BK_{ca} channel is widely distributed in VSMCs, whereas IK_{ca} and SK_{ca} channels are abundantly expressed in endothelium (Ding et al., 2002, Eichler et al., 2003, Jakala et al., 2009, Marchenko and Sage, 1996). The electric conductance for BK_{ca} , IK_{ca} , and SK_{ca} channels ranges between 100-300, 25-100, and 2-25 pS, respectively. In VSMCs, the BK_{ca} channel is voltage and Ca^{2+} -dependent, it will be activated by the increased intracellular Ca^{2+} concentration, which allows the efflux of K^+ ions from the cytosol, hence creating hyperpolarization and the closure of the Ca^{2+} channels, which results in vasodilatory effect (Feletou and Vanhoutte, 2005, Gautam et al., 2006). Furthermore, BK_{ca} channel can be indirectly stimulated by the activation of PKA and PKG (Robertson et al., 1993, Scornik et al., 1993). IK_{ca} and SK_{ca} channels are slightly different from BK_{ca} channel by which they are voltage-insensitive (Barfod et al., 2001, Hirschberg et al., 1998, Xia et al., 1998), but highly sensitive to the Ca^{2+} and calmodulin concentration in cytosol (Garcia Pascual et al., 1995, Schumacher et al., 2001). The commonly used selective antagonist for BK_{ca} , IK_{ca} , and SK_{ca} are iberiotoxin, clotrimazole, and apamin, respectively.

1.5.1(b) Voltage-activated K^+ channel (K_v)

In vasculature, the activation of K_v channel is strictly dependent on the voltage changes across the membrane and is functionally correlated with the voltage-operated Ca^{2+} channel (VOCC) in maintaining the membrane potential of VSMCs. K_v channel will reverse the depolarizing state of the membrane potential back to steady state (Nelson and Quayle, 1995). Typically, there are two major subunits for K_v channel

which are the alpha subunits, which can be further grouped into 12 subclasses, and the beta subunits. They are functionally activated to hasten the efflux of K^+ from the cytosol out to the exterior, hence a more rapid increase of repolarizing current, with less activation of Ca^{2+} channels (Jakala et al., 2009, Robertson and Nelson, 1994, Yildiz et al., 2013). In addition, the cAMP-dependent PKA can indirectly increase the amplitude of the K_v currents (Aiello et al., 1995) and inhibited by the activation of PKC (Cole et al., 1996). The commonly used inhibitor for its mechanism study is 4-aminopyridine (4-AP).

1.5.1(c) Inwardly-rectifying K^+ channel (K_{ir})

This channel contains pore domain which is homologous to K_v channel, and therefore it has been classified as one of the member in K_v channel. The K_{ir} channel needs to bind with PIP_2 to be activated, and functionally prefer the inward flow of the K^+ ions rather than outward, hence hastening the recovery of the membrane potential back to resting state (Feletou and Vanhoutte, 2005, Ganong, 1993, Tucker and Baukrowitz, 2008). The activation of this channel in endothelium will contribute as EDHFs for inducing the relaxing of VSMCs (Edwards et al., 1998). The only different of K_{ir} channel compared to other K^+ channels is it will only be activated when the membrane potential has reached hyperpolarization state, then allowing the influx of K^+ ions into the cytosol to reach resting potential (Edwards and Weston, 1995). The only selective antagonist used for the K_{ir} channel study is barium chloride ($BaCl_2$).

1.5.1(d) ATP-sensitive K^+ channel (K_{ATP})

This channel will be activated by increasing intracellular ADP and decreasing intracellular ATP, also called ATP-sensitive channel and is located in VSMCs (Ashcroft and Ashcroft, 1990, Boyd et al., 1990, Ko et al., 2008, Standen et al., 1989). In resting potential, K_{ATP} channel acts as a weak inwardly rectifying K^+ channel, thus it was

classified as a member in K_{ir} channel family. However, once K_{ATP} channel is activated, it will produce K^+ efflux from the cytosol to maintain a negative resting potential, hence causing vasodilatory effect (Jakala et al., 2009). The commonly used selective K_{ATP} channel inhibitor for mechanism study is glibenclamide.

1.5.2 Calcium channels (Ca^{2+})

The Ca^{2+} ion-linked receptor is selectively permeable for Ca^{2+} ions and allows its entrance into the cytosol, hence creates depolarization, and induces vasoconstriction. Typically, there are two different types of Ca^{2+} channel such as VOCC and receptor-operated Ca^{2+} channels (ROCC). Generally, there are two ways to increase the cytosolic Ca^{2+} concentration which are through 1) the influx of Ca^{2+} ions from exterior or 2) intracellular release of Ca^{2+} from the SR store. Ca^{2+} ion is one of the most crucial second messengers in vascular tone regulation. The intracellular increase of Ca^{2+} concentration will cause membrane depolarization and allow the up-regulation of Ca^{2+} -calmodulin complexes. In the cross-bridge cycle in VSMCs, the activated calmodulin will stimulate the MLC kinases (MLCK) to phosphorylate the MLC at serine residue-19 to form a cross-bridge with the actin filament, and again form actin-myosin protein (AMP), hence causes the VSMCs contraction via the sliding filament mechanism (Gao et al., 2003, Jakala et al., 2009, Marchenko and Sage, 1996, Webb, 2003). In addition, there is another enzyme in this cross-bridge, the MLC phosphatases (MLCP) which can dephosphorylate the MLC in order to terminate the smooth muscle contraction.

1.5.2(a) Voltage-operated Ca^{2+} channel (VOCC)

In vasculature, VOCC is one of the most important receptors used to control the vascular tone by maintaining the membrane potential of the VSMCs. Here, the VOCC is normally referred to as the L-type Ca^{2+} channel that is in VSMCs. In normal

physiological condition, the concentration of the Ca^{2+} ions outside the cell is around 3-4 mM which is thousand-fold compare to inside the cell, which is normally kept at or below 100nM (McFadzean and Gibson, 2002). Therefore, once the membrane potential reached depolarization stage, the Ca^{2+} ions will rush into the cytosol from the outside via VOCC, hence results in vasoconstriction (Goodman et al., 2001). It is functionally correlated with K_v channel in controlling the membrane potential. The commonly used antagonist for this channel mechanism study is nifedipine due to its highly vascular selectivity (Furberg et al., 1995).

1.5.2(b) Receptor-operated Ca^{2+} channel (ROCC)

The calcium cannot only enter the VSMCs by Ca^{2+} influx via VOCC, but also through intracellular Ca^{2+} release via the ROCC that could lead to the membrane depolarization (Berridge, 1997, Gibson et al., 1998). The ROCC normally refer to certain members of GPCRs that are capable of inducing the intracellular release of Ca^{2+} ions from the SR store into the cytosol by producing its second messenger (Landsberg and Yuan, 2004, McFadzean and Gibson, 2002). Typically, there are at least three types of receptors that are categorized as ROCC such as IP_3R , RyRs, and store-operated Ca^{2+} channels (SOCC).

1.5.2(b)(i) Inositol triphosphate receptor (IP_3R)

The IP_3R is located on the surface of the SR, which will be activated by the second messenger, IP_3 that is produced by activated $\text{G}_q\alpha$ protein-coupled receptors. It is the main site for the intracellular Ca^{2+} release from the SR store into the cytosol which leads to an increase in the formation of Ca^{2+} -calmodulin complexes (Putney et al., 2001). Regarding to this mechanism study, the selective IP_3R blocker, 2-aminoethoxydiphenyl borate (2-APB) is commonly used.

1.5.2(b)(ii) Ryanodine receptor (RyRs)

This receptor is less likely distributed in vascular, however, it plays minor role in controlling the intracellular release of Ca^{2+} ions into the cytosol by using calcium-induced calcium release (CICR). The increasing Ca^{2+} ions concentration in cytosol will trigger the RyRs to release more Ca^{2+} ions from the SR store which subsequently causes a transient Ca^{2+} spark, which is important for muscle contraction.

1.5.2(b)(iii) Store-operated Ca^{2+} channel (SOCC)

The primary function of SOCC is to refill Ca^{2+} ions, and it is known as capacitative-dependent calcium entry channel whereby its activation across the plasma membrane will occur with the depletion of the Ca^{2+} stores (Feletou and Vanhoutte, 2005, McFadzean and Gibson, 2002). The sacro/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) is the only specialized pumps used to transport the Ca^{2+} ions from the extracellular into the SR store to re-accumulate the calcium concentration back to the range between 0.5-1 mM. In the SR store, the Ca^{2+} ions will bind to calsequestrin with the purpose of decreasing the free mobile Ca^{2+} ions in the SR, hence more calcium can be stored (Chemaly et al., 2013, Swietach et al., 2008, Wood, 2016). The selective SOCC blocker, gadolinium (Gd^{3+}) is usually accompanied with the use of the selective blocker of SERCA, thapsigargin (Rogers et al., 1995) during the mechanism study of SOCC pathway.

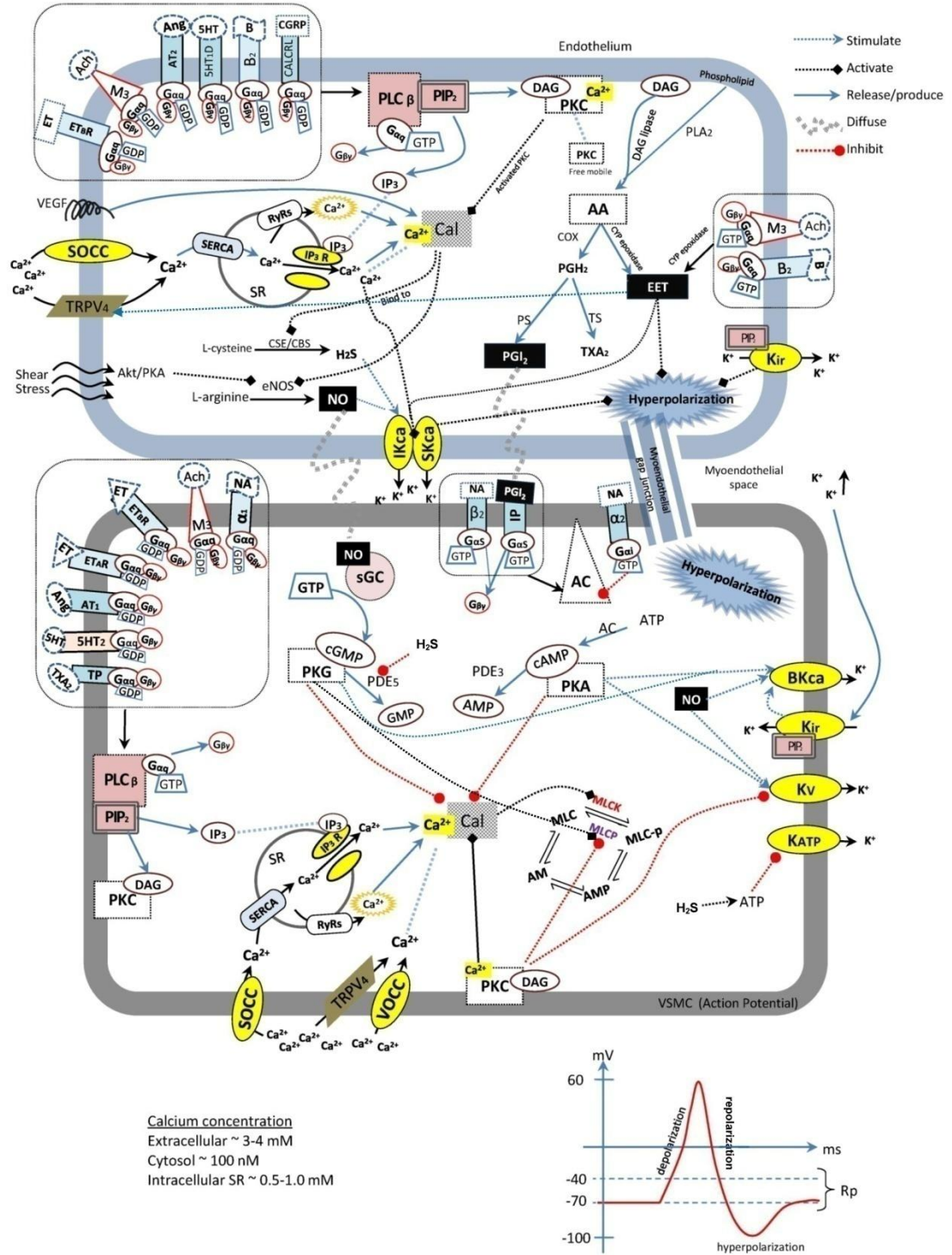


Figure 1.1: Overall signaling mechanism pathways that happen in vascular endothelium and vascular smooth muscle cells which mediate vascular tone regulation.

In summary, there are plenty of signaling mechanism pathways mediating vascular tone regulation as shown in Figure 1.1. However, in the signaling mechanism studies of vasodilatory effect of drugs, there are a few major vasodilation-predominated signaling pathways that need to be studied and investigated such as NO, PGI₂, sGC, cGMP, muscarinic receptors, β_2 -adrenergic receptors, K_{ca}, K_v, K_{ir}, K_{ATP}, VOCC, and IP₃R. According to the literature, all these pathways were frequently studied in the *in vitro* antihypertensive pharmacological research related to vasculature (Loh et al., 2016, Rameshrad et al., 2016). All these signaling mechanism studies have been included in this thesis as well.

1.6 Problem Statements

Despite there are varieties of synthetic antihypertensive drugs available in the current market, there are often many scientific reports on their relatively low efficacies in term of monotherapy as well as undesired chronic adverse effects (Düsing, 2010). According to the trend of antihypertensive drugs evolution, the classes of antihypertensive drugs used clinically often changes from year-to-year with the purpose of achieving the targeted blood pressure range. For instance, the development of antihypertensive drugs have evolved in such an order: Rauwolfia alkaloid, reserpine, thiazides, β -blockers, calcium-channel blockers (CCBs), α -blockers, angiotensin-converting-enzyme inhibitor (ACEI), angiotension II-receptor blockers (ARBs), and direct renin inhibitor (DRI) Aliskiren. However, according to the Clinical Practice Guidelines in Management of Hypertension (4th edition) in Malaysia, the favored choice of first line monotherapy has included only five classes from the aforementioned including ACEI, ARBs, CCB, diuretic-thiazide, and β -blockers, and report stated that only 35% of the patients' blood pressure was successfully controlled by treatment (MOH, 2013). Therefore, combination

therapy is often preferred and was claimed to be more effective in achieving the target blood pressure. At the same time, approximately 60% of the hypertensive patients in Europe countries require ≥ 2 antihypertensive agents to reach the desired blood pressure (MOH, 2013). Because of this, the US, European Society of Hypertension (ESH) and European Society of Cardiology (ESC) guidelines have provided a few options for two drugs combinations such as the combination of diuretic with either CCBs or ACEI/ARBs, while only a single remedy for three drugs combination such as ACEI/ARBs with CCBs plus a diuretic (Düsing, 2010). Thus, the discovery of new antihypertensive drugs is still a hot topic in the field of pharmaceutical research.

From the perspective of traditional Chinese medicine (TCM), it employs a syndromatic treatment system which treats diseases based on its syndromes. Based on the principles of TCM, hypertension is a multi-syndromic disease that is caused by phlegm fluid retention, fire, and deficiencies syndromes. The first syndrome is fire syndrome that could further categorized into four types such as liver fire, heart fire, stomach fire or intestine fire. The second syndrome is the phlegm-fluid retention syndrome. The third syndrome is deficiency syndrome that normally happens in the spleen or the kidney (Qian et al., 2003, Wang and Xiong, 2013). Since ancient times, TCM herbs have been shown to be capable of providing extraordinary therapeutic effect in the treatment of hypertension based on their holistic benefits along with insignificant arise of safety concern. Moreover, the combination of TCM herbs could possibly enhance the therapeutic effect of the main herb in eliciting its vasodilatory effect. These phenomena could be attributed to the variety of vasoactive components present in the combined TCM herbs, which could tackle hypertension through multiple signaling mechanism pathways simultaneously.

In this study, five TCM herbs that are frequently used as clinical prescriptions to counteract different syndromes caused by the hypertension which includes *Gastrodia elata*, *Uncaria rhynchophylla*, *Pueraria thomsonii*, *Panax notoginseng*, and *Alisma orientale* were selected for antihypertensive drugs research by using orthogonal stimulus-response compatibility group studies with $L_{25} (5^5)$ formulation. The high performance thin layer chromatography (HPTLC) and Fourier transform infrared (FTIR) spectroscopy identification methods were used to authenticate the fingerprints of the five herbs by comparison to their respective marker compounds before the experiments started. The vasodilatory effects of formulated combined herbs were determined by using *in vitro* aortic rings assay, followed by oral administration to the spontaneous hypertensive rats (SHRs) for 28 days consecutively in different dosages along with toxicology study. The formulated combined herbs was expected to exert strongest vasodilatory effects using holistic mechanism of actions compared to single herbal extract, as well as to exhibit a strong antihypertensive effects in *in vivo* whilst without causing adverse effect in animal models.

1.7 Objectives

The main objectives of present study were

- a) To investigate the vasodilatory effects of the most potent extracts from *G. elata*, *U. rhynchophylla*, *P. thomsonii*, *P. notoginseng*, and *A. orientale* using *in vitro* aortic rings assay.
- b) To determine the optimum ratio of combining five most potent solvent extracts from five TCM herbs by using $L_{25} (5^5)$ formulation orthogonal stimulus-response compatibility group studies (named as F1).

- c) To determine the most suitable solvent to extract *U. rhynchophylla*, *P. thomsonii*, *P. notoginseng*, and *A. orientale* that prepared in optimized ratio (F1) to exert the highest vasodilatory effect using *in vitro* aortic rings assay.
- d) To study the mechanism of action of vasodilatory effects for the ratio optimized combination of five TCM herbs (F1-2) using *in vitro* aortic rings assay, and partial characterization on its vasoactive components using tri-step Fourier transform infrared spectroscopy and high performance thin layer chromatography.
- e) To determine the antihypertensive and toxicity effects of F1-2 via *in vivo* study on spontaneous hypertensive rats.

CHAPTER 2

HERBAL AUTHENTICATION USING HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY AND TRI-STEP FOURIER TRANSFORM INFRARED SPECTROSCOPY

2.1 Introduction

As aforementioned, the five TCM herbs which include *G.elata*, *U. rhynchophylla*, *P. thomsonii*, *P. notoginseng*, and *A. orientale* were selected for this antihypertensive drug research based on their own capability to cure the specific syndromes caused by the hypertension. Before the experiment carried put, the authenticity of the five purchased raw herbs was assured macroscopically upon purchasing from the local medical hall by the TCM's expert, Dr. Yam Mun Fei. After that, the fingerprints of five herbs were identified by using high performance thin layer chromatography (HPTLC) and tri-step Fourier transform infrared (tri-step FTIR) spectroscopy methods.

2.1.1 High-performance thin layer chromatography (HPTLC)

Basically, all herbs may contain highly complex mixtures of compounds that cover a wide range of substance classes and they are used to exhibit natural variability. Therefore, the choice of selecting an appropriate analytical method is crucial in order to achieve highly reliable and reproducible results, both in qualitative and quantitative manners. HPTLC fingerprint is one of the ideal analytical methods that can be used to identify the complex herbal entities (Sai Saraswathi et al., 2017). Fundamentally, HPTLC is the improved form of TLC, by using the same principles, the mobile phase promotes the separation of compound mixtures by differential migration according to

their polarity across the stationary phase, subsequently the TLC plate will be examined under UV light with different wavelength and the distances travelled by the separated substances will be calculated as retention factor (R_f). However, HPTLC provides extra credits by providing enormous flexibility in the selection of the parameters for different analysis, enhanced resolution, automation steps, and highly accurate quantitative measurements. HPTLC is known as a powerful and rapid analytical tool due to its high flexibility, precision, and accuracy (Maldini et al., 2016). The automation steps of the HPTLC has tremendously enhanced the reproducibility of the quantitative results, and thus it was proposed to be included in various pharmacopeia (Gün et al., 2014, John et al., 2015, Motisariya et al., 2013, Pikul et al., 2013). In this section, the HPTLC fingerprints of each raw herb were compared to the fingerprints of their standard herbs that were purchased from National Institutes for Food and Drug Control, China, as well as compared with their marker compounds that were purchased from Chengdu Biopurify Phytochemicals Ltd, Sichuan, China in order to prove the authenticity of the raw herbs.

2.1.2 Tri-step Fourier transform infrared spectroscopy (tri-step FTIR)

The principles applied in tri-step FTIR is same with FTIR whereby it measures the infrared radiation absorbed or emitted by the substances which normally appears in mid-IR ($5000\text{--}400\text{ cm}^{-1}$) or near-IR region ($10000\text{--}4000\text{ cm}^{-1}$), and rarely in far-IR region (200 cm^{-1}), and the resulting absorption peak appeared in the spectrum corresponds to the bonds present in the molecules of the substances. Tri-step FTIR consists of three types of identifications methods such as conventional FTIR (1D-IR), second-derivative IR (SD-IR), and 2D-correlation IR (2D-IR) spectroscopy. Tri-step FTIR is easy and rapid, and is used to provide highly reproducible fingerprint results which make it the golden identification method in determining the authenticity of TCM materials. The

main advantage of tri-step FTIR is that the sample does not need to undergo any destructive chemical or physical perturbation processes such as fractionation which could cause the original samples to behave differently, and therefore the most complete and true information among the relationship between different chemical compounds in each herb can be obtained (Sun et al., 2011).

During the spectra interpretation, some of the characteristic peaks would be too small due to the low concentration of a particular chemical constituent within the sample, or had been unintentionally masked by bigger peaks, thus those peaks were invisible for interpreting in the 1D-IR spectra. Therefore, the SD-IR spectroscopy with its higher resolution can be used to solve this problem by emphasizing the hidden peaks in the SD-IR spectra (Sun et al., 2003). Moreover, the 2D-IR spectroscopy is the next step to compensate the drawbacks of other analytical systems by exposing the samples to external perturbation, and therefore altering the intra- and intermolecular relationship of the chemical constituents within the samples. Subsequently, the relationship between functional groups could be analyzed from the dynamic spectra obtained (Li et al., 2014, Li et al., 2015). In this section, the 1D-IR spectra of raw herbs were compared to the standard herbs that were purchased from National Institutes for Food and Drug Control, China to prove their authenticity.

2.2 Methodology

2.2.1 Herbs and chemicals

The *G. elata*, *U. rhynchophylla*, *P. thomsonii*, *P. notoginseng*, and *A. orientale* raw herbs were purchased from the local medical hall and macroscopically authenticated by the expert of TCM, Dr. Yam Mun Fei. The *G. elata*, *U. rhynchophylla*, *P. lobata*, *P. notoginseng*, and *A. orientale* standard herbs were purchased from National Institute for

Food and Drug Control (Beijing, China). The marker compounds such as gastrodin, rhynchophylline, isorhynchophylline, puerarin, ginsenoside Rb1, ginsenoside Rg1, and alisol B acetate were purchased from Chengdu Biopurify Phytochemicals Ltd (Sichuan, China). HPTLC 60G F₂₅₄ plate and potassium bromide (KBr) were bought from Merck (Germany). Chloroform, ethyl acetate, formic acid, petroleum ether, and ethanol were purchased from QReC (New Zealand). Methanol was purchased from J. T. Baker (USA).

2.2.2 High performance thin layer chromatography

All samples were freshly prepared on the day of analysis. *G. elata*, *U. rhynchophylla*, *P. thomsonii*, *P. notoginseng*, and *A. orientale* raw and standard herbs were prepared in concentration of 100 mg/ml by dissolving in methanol separately. The marker compounds preparations for each herb were described at 2.2.2(a)-(e). After that, all the samples were centrifuged at 3500 rpm for 15 min at room temperature, and the supernatant was transferred into vials prior to analysis. These experiments were carried out by using the HPTLC developed by CAMAG which is equipped with the automatic developing chamber (ADC 2), automatic TLC sampler 4 (ATS 4), TLC visualizer, and TLC scanner 4. All data were interpreted by using VisionCATs software version 2.2.16187.4 (CAMAG, Muttenz, Switzerland).

2 µl of each sample were applied as 8 mm width bands with 11.4 mm distance between bands on 20 X 10 cm HPTLC plate. The bands were applied at 8 mm distance from the bottom edge, and 20 mm distance from left edge. Methanol was used to rinse the spotting needle for three cycles when different samples were applied. The samples spotted TLC plate was transferred to ADC 2 which is equipped with a 20 X 10 cm twin-through chamber containing 25 ml of mobile phase for saturation, and another 10 ml for development. Before the mobile phase developments, the chamber was saturated for 10